DEPOLARIZATION OF FLUORESCENCE DEPLETION

A microscopic method for measuring rotational diffusion of membrane proteins on the surface of a single cell

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1. Introduction

We have developed a new method of measuring the rotational diffusion coefficients (D_R) of membrane proteins. It combines the sensitivity of prompt fluorescence measurements with the long lifetime of triplet states. The sensitivity is about 10^9 greater than that offered by other methods [1–5]. We describe the method and an application to band 3 proteins of erythrocyte ghosts.

2. Materials and methods

2.1. *Principles of method*

The use of triplet probes for the measurement of macromolecular rotations in the μs–ms time range has been reviewed by Cherry [1]. The most general method of triplet detection is indirect, by spectrophotometric observation of ground state depletion. Most triplet probes are fluorescent, and in principle depletion of their ground state could as well be measured by a fluorescence depletion method, in which it is not the absorption of a measuring light beam that is measured, but instead the prompt fluorescence that such absorption excites. The signal of interest is then the depletion of fluorescence caused by ground state depletion. This fluorescence depletion is polarized when induced with a suitable bleaching flash, and becomes depolarized by rotational diffusion of the probe or the molecule to which it is attached. Hence we call the method 'depolarization of fluorescence depletion'. It combines the long lifetime of triplet probes with the high sensitivity of prompt fluorescence.

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2.2. *Instrumentation*

Fig.1 shows the apparatus, which is based on a laser–microscope combination introduced originally for measurement of lateral diffusion coefficients (D_L) by fluorescence photobleaching recovery [6,7]. The important modifications needed for measuring D_R are an acousto-optic modulator and a Pockels cell. The modulator is used to attenuate the laser beam intensity over a 10^4-fold range, with a response time of <1 μs. The Pockels cell is used without polarizers, and it serves to rotate the plane of polarization of the laser beam through 90°, again in <1 μs. Further details are in [8] and the legend to fig.1. The operation of the apparatus is illustrated by fig.2, which shows how the modulated laser intensity at the sample (beam power), the action of the Pockels cell, and the fluorescence photon counter output accumulated in a multichannel scaler (MCS) all vary with time during a typical sweep across the 1024 addresses of the MCS at an address time of, e.g., 10 μs. Initially the laser beam is attenuated some 10^3-fold to a measuring level of a few μW and the undepleted fluorescence signal is recorded for ~0.5 ms. The laser beam is then briefly unattenuated for a bleach period of 2–80 μs, causing about 20–30% ground state depletion of the sample due to triplet formation. The photomultiplier and/or MCS are gated out during the bleach period. The intensity then returns to the original measuring level for several ms, during which time the fluorescence depletion lessens as the ground state is repopulated from the triplet state. Next the measuring intensity falls to the maximally attenuated level, to ensure

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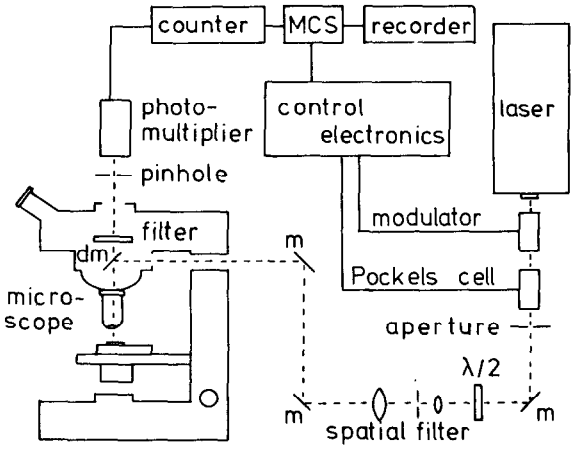

Fig.1. Instrumentation for measuring depolarization of fluorescence depletion. The laser was a 1 W continuous wave argon ion laser (Lexel: model 85-1) with a plane polarized output. The laser beam passes through an acousto-optic modulator (Coherent: analog model 304), a transverse Pockels cell (Electro-Optics Developments: model PC 100/2), a 2 mm aperture to pass only the first-order diffracted beam from the modulator, and a laser beam expander with spatial filter. As described in [8], the output lens of the filter focuses the laser beam to a small spot in the focal plane of the microscope objective. The microscope (Vickers: model M41) combines fluorescence via an epi-illuminator with phase contrast visualization. A temperature-regulated stage was constructed: temperature control was with circulating water, and measurement with a thermistor taped to the slide. The microscope was modified to allow the objective light rays, when undeflected by the eyepiece viewing optics, to come to a focus at a 0.5 mm diam. pinhole mounted in front of a photomultiplier tube (EMI model 9813B with focussing electrode). The pin-hole was adjustable in the objective focal plane an $X-Y$ micrometer mount. A KV 550 barrier filter (Schott) was used for all experiments. Fluorescence was measured by photon counting (Brookdeal model 5C1) and counts were accumulated in a multichannel scaler (Nicolet model 1170 in the MCS model). Minimum address time 1 μs. The control electronics used conventional digital and analogue methods, and served to drive the modulator and the Pockels cells as well as determining timing patterns and interface with the MCS.

full recovery of the ground state. These events occur during the first half of the MCS sweep, typically over 5.12 ms, and are repeated over the second half with one difference: the Pockels cell rotates the polarization plane of the laser beam through 90° for the duration of the bleach period. The depletion signal measured during the first half of the sweep is therefore the parallel signal (||), and that during the second half is the perpendicular (⊥). The sweeps are repeated until a sufficient number of counts per address have been collected.

Three other features of the instrument should be noted. The half-wave plate (or alternatively, a broadband polarization rotator) is used to compensate for optical anisotropies in the microscope, arising mainly at the dichroic mirror. It is essential that the (||) and (⊥) bleaching pulses are of identical intensity at the sample, and the half-wave plate is adjusted until they are. The spatial filter improves the contrast ratio of the acousto-optically modulated beam by ⩾10-fold, from $\geqslant 10^3$–10^4. The pinhole placed before the photomultiplier passes the focussed image of the fluorescent spot, and rejects light from elsewhere [6].

2.3. *Materials*

Eosin- and tetramethylrhodamine isothiocyanate were from Polyscience Inc. (Warrington, PA 18976). Fluorescein- and rhodamine B isothiocyanate were

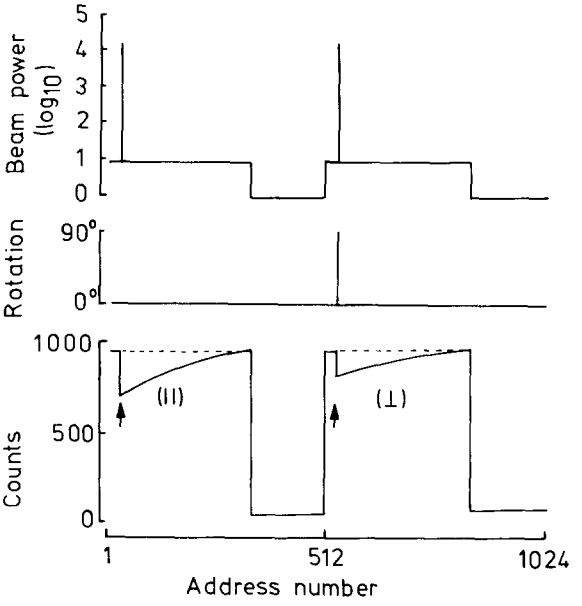

Fig.2. Waveforms illustrating the action of the modulator and Pockels cell. The top trace is the laser beam power in arbitrary units at the sample, plotted logarithmically. The middle trace is the rotation of the laser beam polarization plane at the Pockels cell output. The lower trace is of accumulated fluorescence photon counts in the MCS. The (||) and (⊥) signals are stored in the first and second memory halves, respectively. The signal of interest is the depletion signal, which is the fall of fluorescence from the unbleached level (interrupted lines). The bleaching pulse occurs at the points shown with arrows. Further details are in the text.

from BDH Chemicals Ltd. (Poole, Dorset). Conjugation to albumin was as in [3]. The preparation of erythrocytes, labelling with eosin isothiocyanate and conversion to ghosts was as in [9]. Fresh blood was kindly donated by colleagues.

2.4. *Preparation of anaerobic samples*

The triplet state is rapidly quenched by oxygen, and all samples were prepared anaerobically in a N_2-flushed glove box. In addition, samples contained D-glucose (5 mM), D-glucose oxidase (EC 1.1.3.4, 50 units/ml) and catalase (EC 1.11.1.6, 10^4 units/ml) as an oxygen-scavenging system. After cover slips had been placed on samples on conventional microscope slides they were sealed with quick-setting epoxy resin. For some experiments samples were prepared in bacterial counting chambers of known depth (20 μm): these were sealed with silicone grease.

3. Results

3.1. *Choice of probe*

The choice of probe can be illustrated by reference to the xanthene dyes tetramethylrhodamine, rhodamine B, fluorescein, tetrabromofluorescein (eosin) and tetraiodofluorescein (erythrosin). The quantum yield for triplet formation (ϕ_T) for the last of these, erythrosin, is close to unity [10]. Triplet formation is therefore rapid, some 20–30% conversion in 2–3 μs when a laser beam of 5–10 mW is focussed onto a spot about 20 μm in diameter. However, the fluorescent yield ϕ_F is low, about 0.02 [10], and the fluorescent signal excited by the measuring intensity is weak. Increasing the measuring intensity to improve the signal is ultimately limited because measurement must not cause significant further ground state depletion throughout the measuring period. At the other extreme, ϕ_T for the rhodamine dyes in aqueous solution is about 10^{-3} (unpublished) whereas ϕ_F can approach unity. Consequently the ground state of the rhodamine dyes is depleted significantly only with higher laser powers (20–30 mW at 514 nm), smaller spot diameters (2.5 μm) and longer bleach duration (10–50 μs). Nevertheless, we calculate from [11] that such conditions at a cell surface with 5×10^3 rhodamine molecules/μm^2 would not raise the temperature of the membrane locally by $>0.1°C$. The measuring intensity for rhodamines can be high, because ϕ_T is low. From these considerations we can define the ratio ϕ_F/ϕ_T as a figure of merit that indicates the likely sensitivity of a dye for the fluorescence depletion method. Thus the best on this basis are the rhodamines ($\phi_F/\phi_T = 10^3$) folllowed by fluorescein ($\simeq 5 \times 10^2$), eosin, ($\simeq 1.0$) and lastly erythrosin ($\simeq 0.02$).

The probe should exhibit photochemical stability in order to achieve detection sensitivity through repetitive sweeps and summation of photon counts. We found that fluorescein became irreversibly bleached despite anaerobic conditions in just a few score of sweeps, and was therefore a poor probe. Eosin and rhodamine B were found to be reasonably stable, and tetramethylrhodamine most stable of all. In practice, usable fluorescence depletion signals of 20–30% depletion and about 10^3 counts/10 μs address were obtained in 2^{13}–2^{15} sweeps from about 10^5 molecules of eosin- and 10^3 molecules of tetramethylrhodamineisothiocyanate conjugated to albumin or erythrocyte ghost membranes. Fluorescence depletion signals were not observed if oxygen was present in the samples; this observation confirms the correctness of assigning the depletion signals to triplet formation.

3.2. *Rotational mobility*

Fig.3 shows the fluorescence depletion signals arising from eosin either fully immobilized in poly(methyl methacrylate) or attached to albumin in a viscous medium. As expected, the fluorescence depletion signal for the immobilized eosin showed a marked time-independent anisotropy, with $r \simeq 0.18$. In general we observe r-values for eosin immobilized in solid solution of 0.18–0.28; the higher values require careful attention to the settings of the Pockels cell and half-wave plate, and agree well with values obtained by linear dichroism [12] or phosphorescence [5]. Also as expected the sample of eosin bound to albumin in a viscous medium (3000 cP) showed an initial anisotropy ($r = 0.20$) declining to zero with a relaxation time of about 200 μs. Again this result is similar to those reported using phosphorescence [3] or linear dichroism [13] at 10^6–10^9-fold lower sensitivity.

We were unable to detect anisotropy of fluorescence depletion when eosin–albumin conjugate was examined in aqueous buffer, and this is in keeping with the fast isotropic rotation well below the time resolution of our apparatus. The fast isotropic rotation of fluorescent triplet probes in aqueous buffer was used as the basis for controls to ensure that the

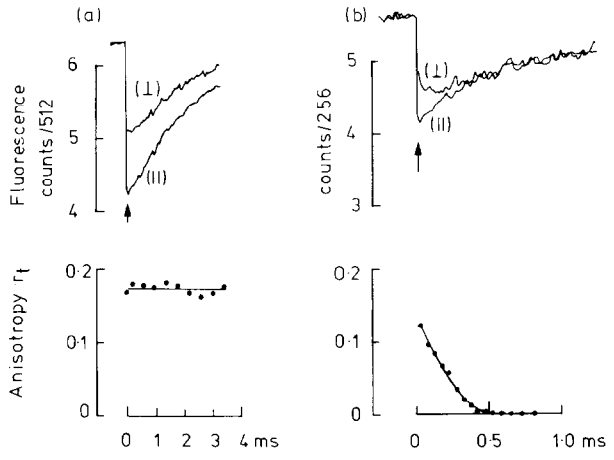

Fig.3. Polarized fluorescence depletion measurements on (a) eosin in solid solution and (b) eosin attached to albumin in viscous solution. The upper traces are fluorescence photon counts per address, the lower traces are the anisotropy (r_t) calculated after smoothing the fluorescence curves. Eosin in a poly(methyl methacrylate) block was used in expt. (a), with a 20 μs bleach period and a X20 long working distance microscope objective. 2^{11} sweeps were accumulated at 10 μs/address. In expt. (b), eosin isothiocyanate conjugated to bovine serum albumin (1:1 molar ratio) was dissolved in 96% (w/w) glycerol (29 μM eosin) and examined with a X20 lens in a 20 μm thick film at 2°C. The spot was 22.5 μm diam., the bleach period 20 μs, and 2^{13} sweeps were collected at 5 μs/address.

(∥) and (⊥) fluorescent depletion signals were identical when sample anisotropy was absent.

3.3. *Rotational mobility of band 3 protein in erythrocyte ghosts*

The rotation of the anion translocator (band 3 protein) in erythrocyte ghosts has been studied both by linear dichroism [9] and phosphorescence [2]. This protein is present at high concentration (1.1×10^6 copies/cell [14]) and labels rather specifically with eosin isothiocyanate [9,14]. It is reported to exhibit two rotational motions in the μs—ms time range [9]: at 37°C, about 25% of the flash-induced anisotropy exhibits a decay time of 150 μs, another 50% has a decay time of 3.4 ms, and there is a residual anisotropy of about 25% (i.e., $r_\infty/r_0 = 0.25$). It is unknown [1] whether the reported anisotropy decay times reflect, respectively, D_R^{-1} or mainly $(4D_R)^{-1}$. We have used eosin isothiocyanate to label band 3 of erythrocyte ghosts, and fig.4 shows fluorescence depletion traces for a large field (diam. 25 μm) containing about 7 adjacent ghosts, and also traces for a small spot (diam. 2 μm) focussed on the top surface of a ghost. With the whole ghosts the anisotropy of fluorescence depletion decayed from an r_0 value of 0.17 in fast and slow phases towards a residual anisotropy of 0.05. Although noise prevents precise values being calculated for these decays of anisotropy, they are clearly similar to those reported for linear dichroism measurements made on about 10^9 erythrocyte ghosts in the optical path of the laser beam [9].

With a single ghost and small laser spot (fig.4) the anisotropy decayed not only completely, but about 4-fold faster. The absence of residual anisotropy using a small spot on a single ghost was as expected from the optical geometry (see [15]). Furthermore, the much faster decay of anisotropy with the orientated single cell indicated that in the randomly orientated

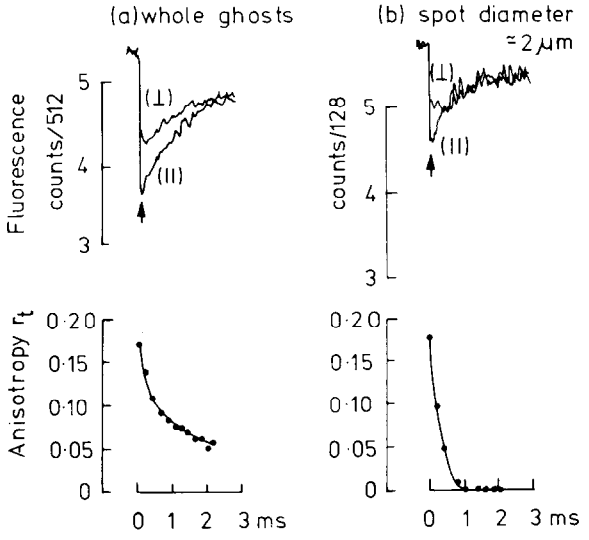

Fig.4. Rotational mobility of band 3 protein in erythrocyte ghosts. Fluorescence depletion measurements were made on a thin (7–10 μm) film of erythrocyte ghosts at 37°C, labelled with eosin isothiocyanate to give 1.7–3.0 nmol eosin/mg ghost protein or $6–11 \times 10^5$ molecules/ghost assuming that 1 ghost has 6×10^{-10} mg protein [14]. For expt. (a) a X20 microscope objective (numerical aperture = 0.65) was used and measurement was made from a 25 μm diam. spot which included 5–7 ghosts. For expt. (b) a X40 objective (numerical aperture = 0.71) was used, and the laser beam was focussed to its smaller diameter (~2 μm) on the top surface of a single ghost. The bleach period was 10 μs, with 2^{13} sweeps collected in (a) and 2^{15} in (b) at 10 μs/address. The top traces are of fluorescence photon counts/address, the lower traces are the anisotropy decay curves calculated after smoothing the fluorescence curves.

situation (whole ghosts) the decay was governed by D and not $4D$. Thus we conclude that for eosin isothiocyanate attached to band 3 protein of ghosts, the reported relaxation times for anisotropy decay [9] are equal to D^{-1}, not $(4D)^{-1}$. Unfortunately rhodamine or tetramethylrhodamine isothiocyanate did not specifically label band 3 (unpublished), so we were unable to use the higher sensitivities that would otherwise have been available.

4. Discussion

We have successfully developed a microscopic method for measuring rotational motion of membrane proteins. The method offers many advantages over others; it is highly sensitive, down to 10^3 molecules with tetramethylrhodamine conjugates; it allows a defined and orientated optical geometry to be used with the result that data analysis is simpler and less ambiguous; the range of probes is potentially wide, not limited to those of higher triplet yield; and the apparatus can also be used, with the same probes, to measure lateral diffusion coefficients.

In illustrating the method we have referred mainly to experiments using eosin. The properties of eosin as a probe were well suited to our apparatus (514.5 nm argon ion line), and there are also comparable data available using linear dichroism and phosphorescence. The sensitivity was quite good, about 10^7 molecules studied in the experiments with erythrocyte ghosts and $<10^5$ molecules with the 2 μm diam. spot on a single ghost (fig.4). Tetramethylrhodamine isothiocyanate conjugates give about a 100-fold increase in sensitivity over eosin conjugates for triplet detection by fluorescence depletion, and their triplet state lifetime is longer (3–5 ms). Future developments should include exploration of alternative fluorescent probes, and improvement of the time resolution. The possible applications of the method to cell surface phenomena, cell biology and model membranes are numerous.

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